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PathHunter® eXpress β-Arrestin Human and Ortholog GPCR Assays

User Manual

Simple Solutions for Complex Biology

CONTENTS

APPENDIX A: RELATED PRODUCTS

Description	Catalog Number	For more information, visit:
Control Ligands	Many	www.discoverx.com/pathway_assays/control_ligands.php
Cell Plating Reagents	93-0563R Series	www.discoverx.com/certified/cell_plating_reagents
PathHunter® eXpress β-Arrestin GPCR Assays	Many	www.discoverx.com/gpcrs/express_arrestin.php
PathHunter® eXpress β-Arrestin Orphan GPCR Assays	Many	www.discoverx.com/gpcrs/express_orphan.php
PathHunter® eXpress β-Arrestin Ortholog GPCR Assays	Many	www.discoverx.com/gpcrs/express_ortholog.php

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: Can I use the PathHunter eXpress β-Arrestin GPCR Assays to study novel GPCR Heterodimers?

A: Yes. To perform a heterodimer experiment, transiently transfect the PathHunter eXpress cells with a plasmid expressing an untagged GPCR receptor. Twenty-four hours post-transfection, follow the agonist protocol as described on page 9 and treat the cells with ligand specific to the untagged GPCR and measure effects on the PK-tagged GPCR using PathHunter Detection Reagents. As a negative control for the assay, use cells expressing the single GPCR to deter mine selectively and specificity of your compounds. For these experiments, we recommend use of a lipid-based transfection reagent such as FuGENE® 6 Transfection Reagent (Roche Applied Science, Cat. #11 815 091 001). Visit www.discoverx.com/gpcrs/dimerization for more details.

Q: Can my eXpress assay be run in 384-well format?

A: All PathHunter eXpress assays are optimized and formatted to be run in 96-well plates. Certain assays also perform well in 384-well format but require modifications to the protocol (cell numbers, incubation times, etc.). Please contact Technical Support (techsupport@discoverx.com) for more information.

LEGAL SECTION

This product and/or its use is covered by one or more of the following U.S. patents #6,342,345 B1, #7,135,325 B2, #8,101,373 B2 and/or foreign patents, patent applications, and trade secrets that are either owned by or licensed to DiscoveRx® Corporation.

LIMITED USE LICENSE AGREEMENT

The cells and detection reagents (collectively Materials) purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Protein:Protein Interaction assay (Assay) that employs genetically modified cells and vectors (collectively, the "Cells"), and related detection reagents (the "Reagents") (collectively referred to as "Materials"). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

1. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.

2. The Reagents contain or are based upon the proprietary and valuable know-how developed by DiscoveRx, and the Reagents have been optimized by DiscoveRx to function more effectively with the Cells in performing the Assay. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser's behalf.

3. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.

If the purchaser is not willing to accept the limitations of this limited use statement and/or has any further questions regarding the rights conferred with purchase of the Materials, please contact:

Licensing Department
DiscoveRx Corporation
42501 Albrae Street
Fremont, CA 94538 USA
tel | 1.510.979.1415 x104
info@discoverx.com

For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

INTENDED USE

PathHunter® eXpress β-Arrestin Human and Ortholog GPCR kits are ready to use complete kits that contain everything you need to perform a functional whole cell GPCR assay in live cells but without any cell culture. The eXpress kits include single use vials of frozen cells stably expressing the GPCR of interest, optimized cell plating reagent, chemiluminescent detection reagents and plates*. Simply thaw and plate the pre-validated cells and challenge with compound 24 or 48 hours later. Whether you are measuring one or multiple GPCR responses to compound challenge, the ready-to-assay eXpress format eliminates the need for lengthy, expensive and time consuming cell culture and makes functional testing fast and convenient. Assays are designed for 96-well plate analyses and kits include enough cells and detection reagents for either 100, 200 or 1000 data points.

*Test compounds are not included and must be provided by the researcher.

TECHNOLOGY PRINCIPLE

PathHunter β-Arrestin products monitor GPCR activity by detecting the interaction of β-Arrestin with the activated GPCR using β-galactosidase (β-gal) enzyme fragment complementation (EFC, Figure 1). In this system, the GPCR of interest is fused in frame with the small, 42 amino acid fragment of β-gal called ProLink™ and co-expressed in cells stably expressing a fusion protein of β-Arrestin and the larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). Activation of the GPCR stimulates binding of β-Arrestin to the ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β-gal enzyme. This action leads to an increase in enzyme activity that can be measured using chemiluminescent PathHunter Detection Reagents. Because Arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation.

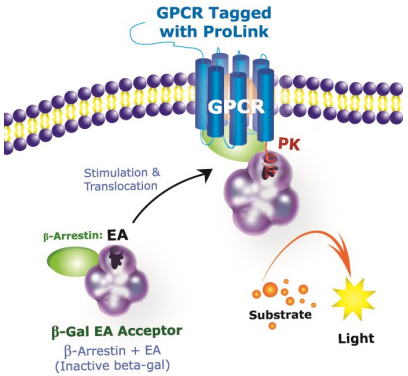


Figure 1. PathHunter® β-Arrestin Assay Principle. Activation of the ProLink-tagged GPCR results in β-Arrestin recruitment and formation of a functional enzyme capable of hydrolyzing substrate and generating a chemiluminescent signal.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: Why do longer incubation times with Detection Reagents lead to a higher signal?

A: The complemented β-galactosidase (β-gal) enzyme is continually turning over the substrate. Theoretically, the signal continues to increase until the substrate is depleted. Therefore, the longer you incubate the reaction, the higher the RLU values.

Q: What is the shelf life of the eXpress kits?

A: We recommend that eXpress kits should be used within 6 months of receipt under proper storage conditions. For short term (2 weeks or less), store eXpress cells at −80°C. For long term storage (more than 2 weeks), store in the vapor phase of liquid nitrogen (N_2). Store the Detection Reagent Kit at −20°C. Refer to the kit label for lot specific expiration date information.

Q: What if my CP reagent changes from a red/pink color to yellow after freezing/thawing?

A: If the CP reagent changes color from red/pink to yellow after thawing, please continue with the assay according to the product insert. We have observed this color change on rare occasions and have confirmed that it does not affect assay performance.

Q: Can I use the PathHunter eXpress β-Arrestin GPCR assay to test inverse agonist compounds?

A: Yes. For compounds that behave as inverse agonists, follow the agonist protocol as described on page 10.

Q: Can the source of my agonist or antagonist compound impact my assay performance?

A: Yes. The vendor/source of compound can impact assay performance dramatically. Compounds can vary in purity from vendor to vendor. In addition, vendors will recommend different diluents (methanol, NaOH, ethanol, DMSO, water), different treatments (boiling, freeze/thaw, etc) or different storage temperatures for the same compound. Each PathHunter eXpress target has been QC tested and validated using a reference ligand. Information on the reference ligand used for each assay (including the vendor source and catalog number) can be found on the cell line specific datasheet. For optimal assay performance, we recommend using control ligands provided by DiscoveRx. Visit www.discoverx.com/pathway_assays/control_ligands.php for the complete DiscoveRx offering.

Q: What Protocol can I use for antibody studies?

A: You should use an antagonist protocol for detection of neutralizing antibodies. Refer to pages 20-23 for more details.

Q: Can I use this assay to test human plasma or serum samples?

A: Yes. PathHunter eXpress β-Arrestin GPCR assays tolerate up to 80% serum or plasma. First, prepare a standard curve of spiked ligand in neat, heparinized plasma (or mouse, human serum). Add samples directly to the cells (no further dilution – 100% plasma in the well). After stimulation, remove the plasma or serum sample and replace with fresh CP reagent before addition of the PathHunter Detection Reagents. It has been shown that EDTA anti-coagulated plasma inhibits EFC and should be avoided for these types of studies.

FREQUENTLY ASKED QUESTIONS

Q: I did not see a signal with my control agonist.
A: There may be differences in agonist purchased from different vendors. Confirm that the control agonist used is the same ligand used in the dose response shown in the provided cell-specific datasheet.

Q: I did not see a response with my compound.
A1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the agonist/antagonist diluent at ≤ 1%.
A2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
A3: Confirm that the cell line responds to the control agonist.

Q: My cells arrived thawed. Can I use them?
A: No. Call technical support for a replacement.

Q: How long is the prepared detection reagent good for?
A: The working detection reagent solution must be used within 8 hours of mixing.

Q: What instruments can I use to read the plates?
A: Any bench top luminometer will work with the PathHunter eXpress β-Arrestin GPCR Assays. Below is a partial list of commercially available luminometers that have been used to validate our assays:

Turner Biosystems: Modulus Microplate
GE Healthcare Life Sciences: LEADseeker™, FarCyte™
BMG Labtech: PHERAstar Plus, LUMIstar Omega
Perkin Elmer: TopCount®, VICTOR II or V, Fusion, LumiCount, EnVision, MicroBeta® (Trilux), ViewLux
Molecular Devices: CLIPR™, LJL Acquest, LJL Analyst, LJL Analyst HT, LJL Analyst GT, Gemini, SpectraMax®, Flexstation™, LMax
Tecan: Ultra Evolution
Beckman Coulter – CRi
Berthold Technologies: Mithras LB 940
Hamamatsu: FDSS6000, FDSS/RayCatcher

Q: How long is the signal stable for?
A: The signal is stable for 24 hours after addition of detection reagent.

Q: My cells are floating after the 48 hours incubation.
A: The cells are not viable, contact technical support for a replacement.

Q: Can I switch plates or should I use the plate provided?
A: You can use any clear bottom white or opaque walled plate.

Q: What if cells are not completely adherent after 24/48 hrs incubation?
A: For certain targets, cells may not be completely adherent after 24 hours, but still greater than 80% viable. Please continue on with the protocol as described in the product insert.

PROTOCOL OVERVIEW

Please read the entire protocol completely before running the assay. Successful results depend on performing these steps correctly. Refer to the cell-line specific datasheet for information on optimized cell plating reagent and reference ligand. For additional information or Technical Support, contact DiscoveRx or visit www.discoverx.com.

The following steps are required to monitor GPCR activity using a PathHunter eXpress β-Arrestin GPCR assay (Figure 2).

1. Thaw and plate frozen, assay-ready eXpress cells (page 9).
2. Dilute and add compounds or antibodies.
3. Perform functional assay in agonist, antagonist or allosteric modulator mode (pages 9, 13 and 17).

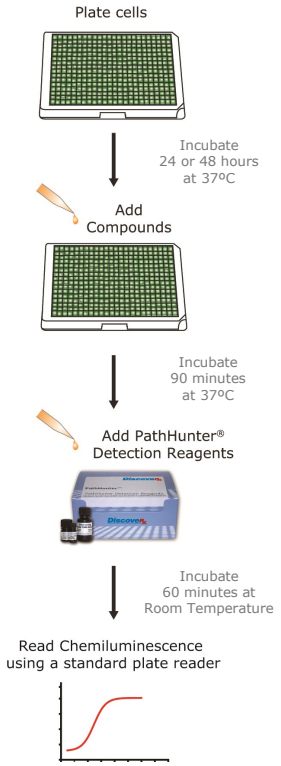


Figure 2. Monitor functional GPCR responses to compound challenge using the fast and simple PathHunter eXpress protocol.

KIT CONTENTS AND STORAGE CONDITIONS

PATHHUNTER EXPRESS KIT COMPONENTS REQUIRE MULTIPLE STORAGE TEMPERATURES. OPEN BOXES IMMEDIATELY AND STORE CONTENTS AS INSTRUCTED.

SHELF LIFE:
Use kit within 6 months from the date of receipt under proper storage conditions.

BOX 1: PATHHUNTER EXPRESS CELLS

STORAGE:
Short term (2 weeks or less): **Store vials at -80°C immediately upon arrival.**
Long term (greater than 2 weeks): **Place vials in the vapor phase of liquid nitrogen (N_2).**

PathHunter eXpress cells arrive frozen on dry ice. Cells are delivered in individual vials containing 1 x 10^6 cells in 100 µL of freezing medium. Each vial contains sufficient cell numbers to generate (1) 96-well microplate prepared at the seeding density described. When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N_2 inside the vial to evaporate and proceed with the thawing protocol (page 8). **Do not touch the bottom of the tubes at any time to avoid inadvertent thawing of the cells. If cells are not frozen upon arrival, do not proceed. Contact technical support.**

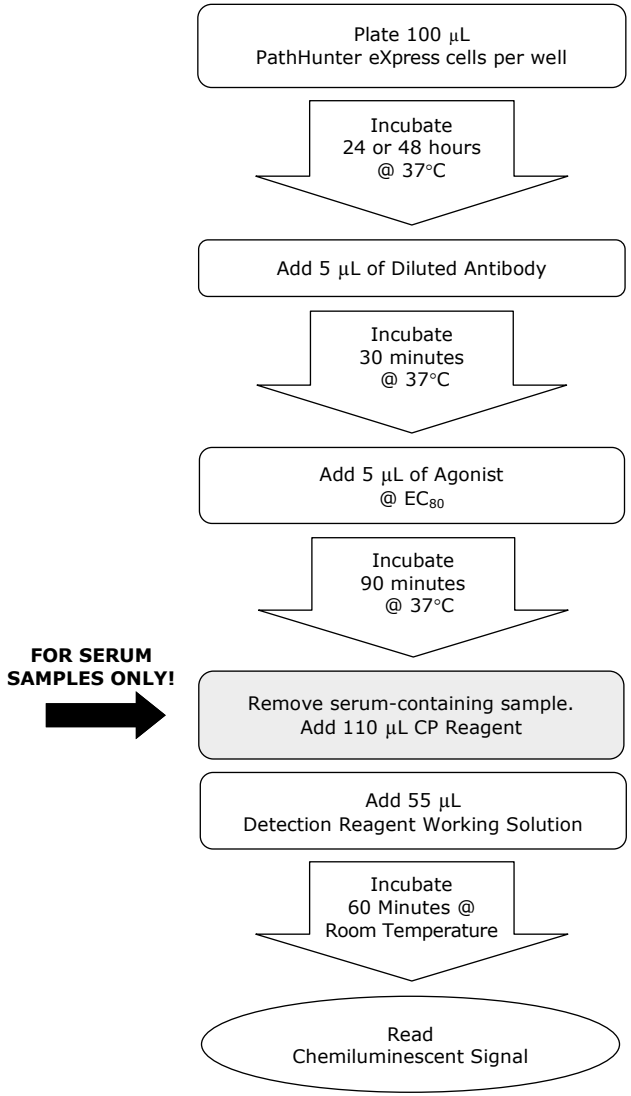
BOX 2: PATHHUNTER DETECTION REAGENT AND CP REAGENT: Store at -20°C

Once thawed, store the Cell Plating (CP) Reagent at 4°C. Avoid multiple freeze/ thaw cycles. In rare instances, the CP Reagent may be yellow in color after thawing. Although this indicates a slight change in pH, continue with the assay as this does not impact assay performance.

Thaw the PathHunter Detection Reagents at room temperature before use, and after thawing, store reagents for up to 7 days at 4°C. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance. Once made, the working solution is stable for 24 hours at room temperature.

BOX 3: 96-WELL TISSUE CULTURE TREATED PLATES: Store at Room Temperature

QUICK-START PROCEDURE: NEUTRALIZING ANTIBODY RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your antibody dose response.

MATERIALS PROVIDED

Description	Contents	Contents	Contents	Storage
Box 1: PathHunter® eXpress β-Arrestin Cells	1 vial 1 x 10^6 cells ea	2 vials 1 x 10^6 cells ea	10 vials 1 x 10^6 cells ea	-80°C (short) Liquid N_2 (long)
Box 2: PathHunter® Detection Reagents - Cell Assay Buffer - Substrate Reagent 1 - Substrate Reagent 2* Cell Plating Reagent‡	100 dp 5.7 mL 1.5 mL 0.3 mL 1 x 20.0 mL	200 dp 9.5 mL 2.5 mL 0.5 mL 2 x 20.0 mL	1,000 dp 57.0 mL 15.0 mL 3.0 mL 2 x 100 mL	-20°C
Box 3: 96-well Tissue Culture Treated Plates	1 plate	2 plates	10 plates	Room Temperature

*Centrifuge vial before opening to maximize recovery.
‡Refer to cell-line specific data sheets for optimized Cell Plating Reagent included with each kit.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required but not provided:

1. Pipettes and pipette tips
2. Tissue culture disposables
3. GPCR control agonist as recommended in the cell line specific datasheet. Visit www.discoverx.com/pathway_assays/control_ligands.php for the complete DiscoveRx offering
4. GPCR Test compound(s)
5. 96-well V-bottom compound dilution plates (DiscoveRx, Cat. #92-0011)
6. Multi-mode or luminescence plate reader.
7. Disposable Reagent Reservoir such as Thermo Scientific, Cat. #8094 or similar

RECOMMENDED MATERIALS

The following products* are recommended:

- CytoTracker™ LDH Quantification Kit (DiscoveRx, Cat. # 92-2002)
- CytoTracker™ Glutathione Quantification Kit (DiscoveRx, Cat. # 92-2003)
- CytoTracker™ DNA Damage Quantification Kit (DiscoveRx, Cat. # 92-2004M)

* Products not available in all countries. Please inquire.

SOLVENTS AND PREPARATION OF COMPOUND DILUTIONS

PathHunter eXpress β-Arrestin assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). As solvents can affect assay performance, optimize the assay conditions accordingly if other solvents or solvent concentrations are required.

To validate each PathHunter eXpress β-Arrestin GPCR Assay, reference ligand was diluted in CP reagent containing appropriate solvent. For preparation of test compounds, we recommend preparing the dilutions using the CP reagent provided in the kit (containing the appropriate solvent). For antibodies or other compounds that may be sensitive to serum and/or other assay components, dilutions can be prepared in either Hanks Buffered Salt Solution (HBSS) + 10 mM HEPES + 0.1% Bovine Serum Albumin (BSA) or OptiMEM® + 0.1% BSA without affecting assay performance.

Some assays require compound incubation times and temperatures that differ from the protocol described in this user manual. **For optimal assay performance, we recommend you perform the assay according to the protocol provided in the cell line specific datasheet.**

USE OF PLASMA OR SERUM CONTAINING SAMPLES

PathHunter eXpress β-Arrestin GPCR assays can be run in the presence of high levels of serum or plasma without negatively impacting assay performance. Standard curves of control ligand can be prepared in neat, heparinized plasma and added directly to the cells (without further dilution, i.e. 100% plasma in the well). After ligand stimulation, the samples should be removed and replaced with fresh CP reagent before the addition of the PathHunter Detection Reagents. Refer to page 20 for more information.

NOTE:
EDTA anti-coagulated plasma samples do not give a positive response in the assay. Therefore, the choice of anti-coagulant treatment is very important.

ASSAY INCUBATION AND CELL PLATING REAGENT REQUIREMENTS

Each PathHunter eXpress β-Arrestin Human and Ortholog GPCR Assay has been validated for optimal assay performance at either 24 or 48 hours post-thaw. Although most targets perform similarly at both time points, for optimal assay performance we recommend you perform the assay according to the protocol provided in the cell line specific datasheet using both the recommended time point and CP Reagent. **Always use the CP Reagent included in the kit and DO NOT substitute from an alternate kit at any time.**

NOTE:
Use special caution when testing multiple targets in the same experiment as targets may have different incubation times and CP Reagent requirements.

d) Add 90 µl of the working concentration of antibody to tube #12.
e) Remove 30 µL of diluted antibody from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 30 µL of diluted antibody from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 7 more times, preparing serial dilutions from right to left across the plate. **DO NOT add antibody to tubes #1 and 2.** These samples serve as the no antibody control and complete the dose curve.
h) Repeat process when testing additional antibodies.
i) Set antibody dilutions aside until they are ready to be added.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes #1-12 to each well according to the plate map on page 20.
5. Incubate for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antibody incubation, determine the EC_{80} concentration of the agonist to be used in the assay. Prepare a **22X** EC_{80} concentration of agonist compound as shown below:

   Example: If the expected EC_{80} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 90 minutes @ 37°C*.
4. If samples do not contain plasma or serum, omit step 5 and proceed directly to the Substrate Preparation and Addition section on page 22.

   NOTE:
   ***Please refer to the cell line specific datasheet for any variation in assay conditions.

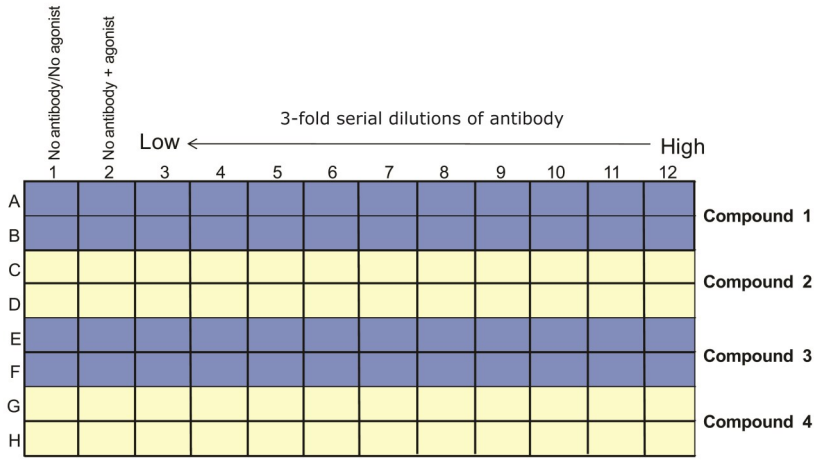
ATTENTION! PLASMA OR SERUM-CONTAINING SAMPLES ONLY

5. After incubation is complete, gently aspirate the plasma or serum-containing samples from the well. Be careful to remove as much sample as possible without disturbing the cell monolayer.
6. Immediately add 110 µL of fresh CP reagent to each well. Proceed to the Substrate Preparation and Addition section on page 22.

   NOTE:
   Additional CP reagent is required for this media exchange step. Please visit www.discoverx.com/certified/cell_plating_reagents for ordering information.

ASSAY PROCEDURE — NEUTRALIZING ANTIBODY DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for detection or anti-GPCR neutralizing antibodies using the PathHunter eXpress β-Arrestin cells and Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing a 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: ANTIBODY PREPARATION AND ADDITION

1. Dissolve antibody in the vehicle of choice at the desired concentration.
2. Prepare 3-fold serial dilutions of antibody in CP reagent containing the appropriate solvent. The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antibody will be used in a final volume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 11-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected IC_{50} value for the antibody (**1100X** IC_{50} would be the final working concentration).

   Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration at 11 µM.

   a) Label tubes 1 through 12.
   b) Add 60 µL of CP reagent containing appropriate solvent to tubes #1-11.
   c) Prepare a working stock of antibody in CP Reagent containing appropriate solvent.

THAWING AND PLATING FROZEN CELLS

The following steps outline the procedure for thawing and plating frozen PathHunter eXpress cells from freezer vials:

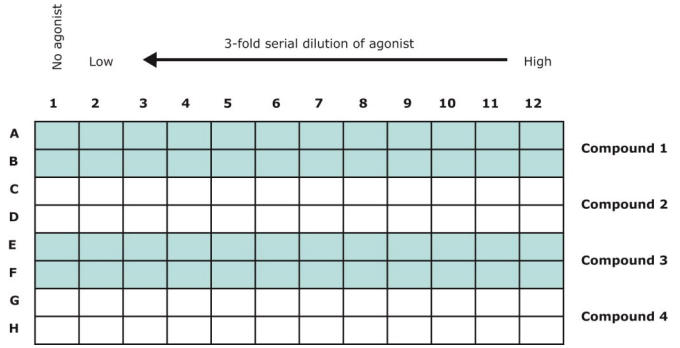
1. Pre-warm CP reagent in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor phase storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   When removing cryovials from liquid N_2, place immediately on dry ice in a covered container. Wait at least one minute before opening for any liquid N_2 inside the vial to evaporate.
3. Place the cell vial(s) ***briefly*** (10 seconds to 1 min) in a 37°C water bath until only small ice crystals remain and the cell pellet(s) is almost completely thawed.
4. Add 0.5 mL of pre-warmed CP reagent to the cell vial. Pipette up and down gently several times to ensure that the cells are evenly distributed.
5. Immediately transfer the cells to 11.5 mL of pre-warmed CP reagent and pour into a disposable reagent reservoir.
6. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
7. After seeding the cells into the microplate, incubate for either 24 or 48 hours at 37°C, 5% CO_2.*

   NOTE:
   *Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE - AGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing agonist assays using the PathHunter eXpress β-Arrestin cells and Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least duplicate wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve agonist compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of agonist compound in CP reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **11X** of the final screening concentration (i.e. 10 µL compound + 100 µL of cells). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 12-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** EC_{50} would be the final working concentration).

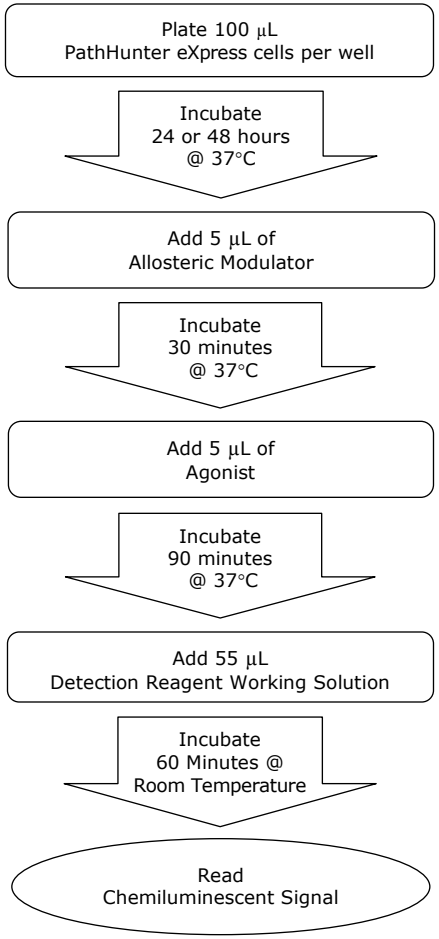
   Example: If the expected EC_{50} is 10 nM, prepare the highest starting concentration at 5.5 µM.

   a) Label tubes 1 through 12.
   b) Add 60 µL of CP reagent to tubes #1-11.
   c) Prepare a working concentration of agonist compound in appropriate CP reagent.
   d) Add 90 µl of the working concentration of agonist compound to tube #12.
   e) Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
   f) With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
   g) Repeat this process 8 more times, preparing serial dilutions from right to left across the tubes.
   h) **DO NOT add agonist compound to tube #1**. This sample serves as the no agonist control and completes the dose curve.
   i) Repeat this process for each compound to be tested.
   j) Set compounds aside until they are ready to be added.
3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL from tubes #1-12 to each well according to the plate map on page 9.
5. Incubate for 90 minutes @ 37°C*.

NOTE:
***Please refer to the cell line specific datasheet for any variation in assay conditions.

3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your allosteric modulator dose response.

QUICK-START PROCEDURE: ALLOSTERIC MODULATOR DOSE RESPONSE



***Please refer to the cell line specific datasheet for any variation in assay conditions.

e) Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 7 more times, preparing serial dilutions from right to left across the plate. **DO NOT add modulator compound to tubes #1 and 2.** These samples serve as the no modulator control and complete the dose curve.
h) Repeat process when testing additional compounds.
i) Set compounds aside until they are ready to be added.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes #1-12 to each well according to the plate map on p.17.
5. Incubate for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the modulator compound incubation, determine the EC_{10} and EC_{90} concentration of the agonist from the agonist dose response curve (described on pages 9-11). Prepare a **22X** EC_{10} concentration (PAM) or **22X** EC_{90} concentration (NAM) of agonist compound as shown below:

   Example: If the EC_{10}/EC_{90} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 90 minutes @ 37°C*****.

NOTE:
*****Please refer to the cell line specific datasheet for any variation in assay conditions.

SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**

SUBSTRATE PREPARATION AND ADDITION

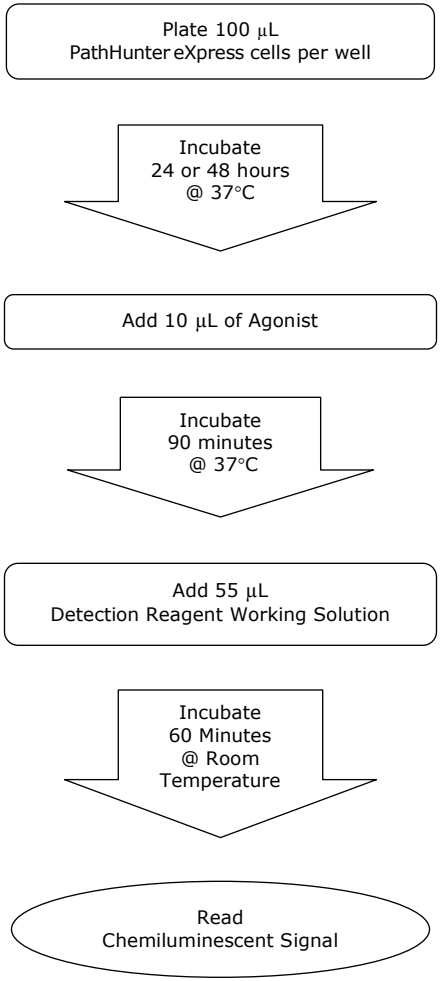
1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

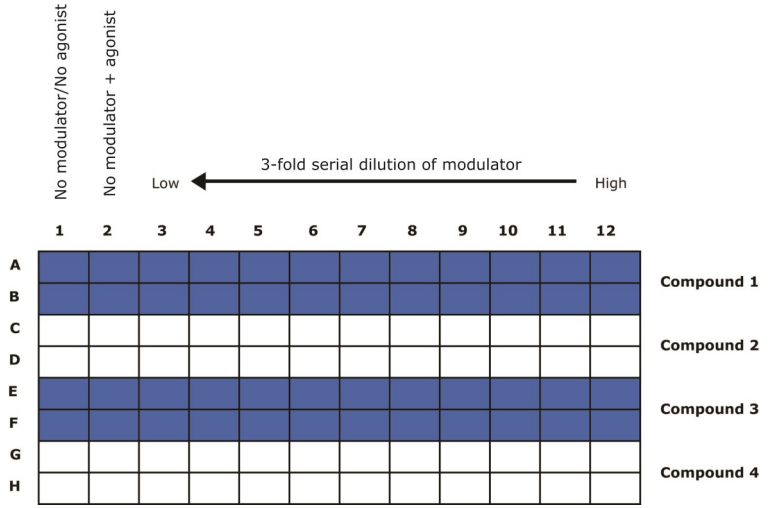
QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE — ALLOSTERIC MODULATOR DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing allosteric modulator assays using the PathHunter eXpress β-Arrestin cells and Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing an 11-point dose curve for each compound concentration using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: MODULATOR COMPOUND PREPARATION AND ADDITION

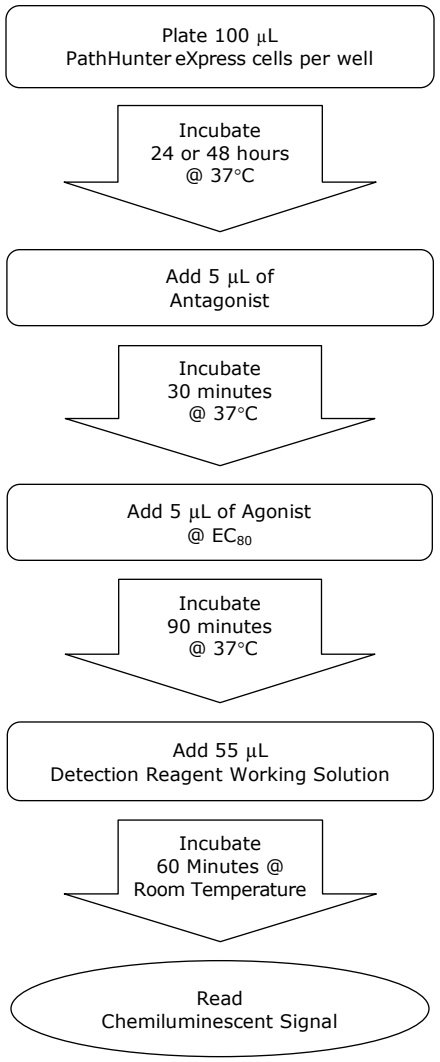
1. Dissolve modulator compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of modulator compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL modulator compound will be used in a final colume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 11-point dose curve serial dilutions: We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** IC_{50} would be the final working concentration).

   Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration at 11 µM. This is the working concentration.

   a) Label tubes 1 through 12.
   b) Add 60 µL of CP reagent containing appropriate solvent to tubes #1-11.
   c) Prepare a working stock of modulator compound in the appropriate CP reagent.
   d) Add 90 µl of the working concentration of modulator compound to tube #12.

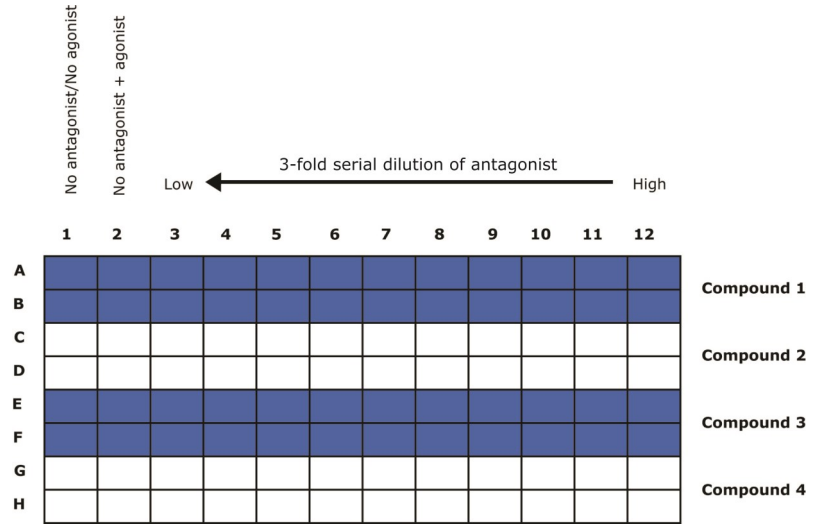
QUICK-START PROCEDURE: ANTAGONIST DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE — ANTAGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing antagonist assays using the PathHunter eXpress β-Arrestin cells and Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing an 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: ANTAGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve antagonist compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of antagonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antagonist compound will be used in a final volume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 11-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** IC_{50} would be the final working concentration).

   Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration at 11 µM.

   a) Label tubes 1 through 12.
   b) Add 60 µL of CP reagent containing appropriate solvent to tubes #1-11.

c) Prepare a working stock of antagonist compound in the appropriate CP Reagent.
d) Add 90 µl of the working concentration of antagonist compound to tube #12.
e) Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 7 more times, preparing serial dilutions from right to left across the plate. **DO NOT add antagonist compound to tubes #1 and 2**. These samples serve as the no antagonist control and completes the dose curve.
h) Repeat process when testing additional compounds.
i) Set compounds aside until they are ready to be added.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes #1-12 to each well according to the plate map on page 13.
5. Incubate for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antagonist incubation, determine the EC_{80} concentration of the agonist from the agonist dose response curve (described on pages 9-11). Prepare a **22X** EC_{80} concentration of agonist compound as shown below:
   Example: If the EC_{80} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 90 minutes @ 37°C*.

NOTE:
***Please refer to the cell line specific datasheet for any variation in assay conditions.

SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your antagonist dose response.